Symmetry breaking in navigating cells

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Chapter I
Introduction

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INTRODUCTION

Cells have the remarkable property to sense and migrate towards extracellular gradients of chemoattractants. This directional movement of cells, also called chemotaxis, is important for almost all organisms. Prokaryotes rely on chemotaxis to sense nutrients, toxins, oxygen levels, pH and osmolarity, while metazoans need chemotaxis for embryonic organization [1], tissue morphogenesis [2] and the immune response by leukocytes [3]. Defects in chemotaxis are associated with the development of various diseases such as cancer [4,5], asthma, arthritis, inflammatory bowel disease and cardiovascular diseases [6]. Therefore, it is important to elucidate the exact mechanisms regulating directional migration of cells.

Dictyostelium as a model organism

The most commonly used model systems for studying eukaryotic chemotaxis are the slime mold *Dictyostelium discoideum* and neutrophils. Although these model systems have clearly distinct roles, they have a highly similar chemotactic behavior [7]. They display strong chemotactic responses, their stimuli are well-defined and they are highly sensitive to even shallow gradients (~ 2% across the length of their body) of chemoattractants. *Dictyostelium* has a remarkable life cycle, consisting of a unicellular and multicellular phase. During the vegetative stage, single cells divide by mitosis as they feed themselves on bacteria. The bacteria secrete folic acid, which is a chemoattractant for the *Dictyostelium* cells. Upon starvation, *Dictyostelium* cells undergo a drastic change in gene expression and enter the multicellular phase of its life cycle, which is characterized by development and cell differentiation. The cells start to secrete cAMP and simultaneously sense cAMP secreted by the neighbouring cells thus forming a gradient of cAMP around the origin point. cAMP synthesis and secretion by *Dictyostelium* cells is an oscillatory process. The outward propagation of cAMP is regulated by extracellular and intracellular phosphodiesterases (PDEs) that degrade the accumulated cAMP. This degradation of cAMP by PDEs serves to maintain the steepness of cAMP gradient thereby limiting the interval and degree of signaling. As a result cAMP waves from the initiation centre are propagated every ~ 6 min. These synchronized oscillatory waves of cAMP results in the formation of streams moving along the gradient towards the centre. After 6 hours aggregates are formed and cells start to differentiate between prestalk cells, prespore cells and anterior-like cells (ALC) depending on cell cycle position and state of nutrition. The tipped mound develops into slugs after 16 hours of

starvation. The anterior region of the slug is occupied by prestalk cells, while prespore cells intermingled with the anterior-like cells are present at the posterior end. Settlement of the slug and the subsequent formation of a Mexican hat, mark the beginning of the culmination stage. The prestalk and prespore cells switch positions at the time of culmination to form a fruiting body comprising of a stalk of dead vacuolated prestalk cells and a spore head called sorus on top made by prespore cells (Fig 1). *Dictyostelium* spores can survive harsh unfavourable conditions. Under better conditions the spores germinate to release single celled amoeba in the vegetative stage, thus completing the life cycle.

We use *Dictyostelium* as model system for studying chemotaxis and development because it is a haploid organism with easy genetics, it has a short and simple life cycle, can easily be grown in large quantity for biochemical experiment, and its complete 34Mb genome is sequenced and available on dictybase [8,9]. Most importantly many molecular features regulating chemotaxis are conserved between *Dictyostelium* and higher eukaryotes [7,10].

Chemotaxis

Chemotaxis is a complex cellular process involving three separate but inter-related events: directional sensing, cellular polarity and cell motility [11]. Cell detects an asymmetric extracellular gradient by sensing the changes in receptor occupancy. Previous studies have shown that *Dictyostelium* can sense the receptor occupancy as a function of space (spatial sensing) and time (temporal sensing) [11]. The shallow extracellular gradient of a chemoattractant is transduced into a steep gradient of intracellular signaling components resulting in selective localization of the signaling molecules to one end of a cell facing the gradient. This process also referred to as symmetry breaking [12,13], induces cytoskeletal rearrangement with F-actin polymerization and pseudopod extension at the side of the cell facing the gradient (leading edge) and myosin II assembly at the back of the cell (lagging edge) [14-16]. Pseudopod formation is biased at the leading edge as it is more sensitive to gradients, while lateral pseudopod formation is suppressed. This results in faster and more directional movement of cell towards the direction of chemoattractant. Cells treated with inhibitors of actin polymerization become round and are incapable to move, but still can sense the direction of the gradient and show symmetry breaking [13,17]. Thus directional sensing and symmetry breaking are independent of cell elongation or cell motility [12].

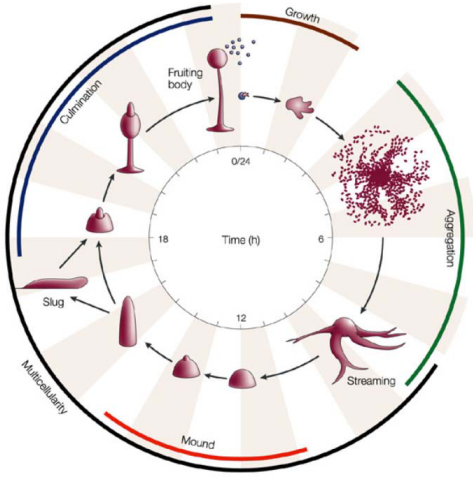

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Figure 1 Life cycle of *Dictyostelium discoideum* During vegetative phase, individual *Dictyostelium* cells feed on soil living bacteria. Upon starvation, cells undergo a drastic change in gene expression and enter the developmental phase where each cell begins to secrete cAMP and chemotax towards cAMP secreted by other cells. This leads to formation of aggregates which differentiate to from slug-like multicellular body which in turn, culminates to form a fruiting body consisting of stalk of vacuolated dead cells and spore head on top.

Gradient detection by GPCR

The initial event in directional sensing of eukaryotic cells is the binding of chemoattractant to G-Protein Coupled Receptors (GPCRs) that are uniformly distributed over the cell surface. GPCRs are ubiquitously present in all eukaryotes with humans having more than 800 GPCRs grouped in five families [18]. The *Dictyostelium* genome encodes for more than 30 GPCRs, including the cAMP receptors, and receptors belonging to GABA, PAF and serotonin families [19]. GPCRs are characterized by seven membrane spanning α-helices with the N-terminal extracellular domain acting as a ligand-binding domain for large ligands such as peptide hormones and small ligands such as cAMP bind in the barrel formed by the seven α-helices. *Dictyostelium* contains four cAMP receptors (cAR1-cAR4) [20,21], that show 54-69% amino acid identity and are

expressed during different stages of the life cycle [22]. cAR1 has the highest affinity for cAMP and it is expressed during early developmental stages. Cells lacking *car1* are insensitive to cAMP, do not show any chemotaxis and fail to initiate development [23]. The expression of cAR1 is low during growth, goes up during early starvation, and decreases again at later stages of development [24]. cAR2 is majorly expressed after 15h of development in anterior prestalk cells. *car2*-null cells do not form tips and development arrests after the tight aggregate stage [25,26]. On the other hand, *car3*-null cells display no characteristic phenotype; they aggregate and develop normally [27]. The level of cAR3 is peaking at mound stage, after 10h of development, and becomes restricted to prespore cells [25]. cAR4 expression starts after tip elongation and drops after the culmination stage. Only 25% of *car4*-null cells are able to form very short fruiting bodies [20]. Although much is known about folate chemotaxis, the folate receptor, through which vegetative *Dictyostelium* cells tracks bacteria has still not been identified.

Heterotrimeric G-proteins

In the absence of ligand, GPCRs are coupled to an inactive heterotrimeric complex consisting of a GDP bound Gα-subunit and a Gβγ dimer. Upon ligand binding, the GPCR gets activated and promotes the dissociation of GDP from the Gα-subunit. The nucleotide-free Gα subunit then rapidly binds GTP which is present in higher concentration in the cytoplasm. Thus GPCRs function as GEFs (Guanine nucleotide Exchange Factors) stimulating the exchange of GDP to GTP on Gα subunit [28-30]. Gα-GTP undergoes a conformational change and dissociates from Gβγ, so that both subunits are free to activate downstream signal transduction pathways. The signal is terminated by the intrinsic GTPase activity of Gα-subunits that mediates the hydrolysis of GTP to GDP. RGS proteins (Regulators of G-protein Signaling) can enhance the intrinsic GTPase activity and thereby accelerate the hydrolysis reaction [31,32]. The Gα-GDP subunit reassociates with Gβγ to form the inactive heterotrimer again [33,34] (Fig 2A).

Dictyostelium contains only a single Gβ [35] and Gγ [36] protein. *Dictyostelium* Gβ shares 60% homology with that of human, *Drosophila* and *C.elegans* [35]. The C-terminal region of Gβ consists of seven WD40 repeats that fold to form a β-propeller structure, which serves as binding site for Gα-subunits and effectors [34,37,38]. The N-terminal region forms a tight coiled-coil structure with Gγ. *gβ*-null cells are viable but fail to aggregate upon starvation

[35]. These cells also lack the ability to chemotax towards folate or cAMP and are defective in cAMP-stimulated activation of guanylyl cyclase and adenylyl cyclase [38]. Consistently, over-expression of a mutant of Gγ, in which four conserved residues (CSVL) at C-terminal have been deleted, results in decreased membrane localization of Gβγ, and subsequently failure of aggregation [36]. This shows that Gβ is essential for almost all chemoattractant-mediated responses and plays a central role in transducing extracellular signals.

The *Dictyostelium* genome encodes for twelve Gα subunits [39], which are most similar to the mammalian Gαi family [39]. They have distinct roles during the life cycle of *Dictyostelium* [40-45] (Table1). Gα2 and Gα4 are essential for chemotaxis towards cAMP and folate, respectively. Gα2 binds to cAR1 and is essential for all cAMP mediated responses [41,46]. Gα4 is expressed during vegetative growth and at low level during early development; the expression increases during mound stage and remains high throughout the development [47]. The subunit Gα4 is essential for folate chemotaxis and during late development stages for the formation of anterior prestalk cells and spores [47,48].

The heterotrimeric G-proteins, are like the GPCRs, localized uniformly on the plasma membrane [49,50]. This ensures the detection of chemoattractant over the whole cell body [49,51]. Previous studies have shown that receptor occupancy and heterotrimeric G-protein activation reflects the steepness of the extracellular gradient of chemoattractant [52]. This suggest that amplification of the extracellular signal and symmetry breaking occurs first downstream of receptor and heterotrimeric G-protein activation [53,54] (Fig 2B).

Small G-proteins

Ras family

One of the first downstream responses of heterotrimeric G-protein signaling is the activation of Ras proteins [55,56]. Ras belongs to the family of small G-proteins, also known as small GTPases that act as molecular switches. G-proteins cycle between an inactive GDP-bound and active GTP-bound state. This cycle is tightly regulated by GEFs which catalyze the exchange of GTP for GDP, thereby activating the Ras protein. GAPs convert the Ras protein back into the inactive GDP-bound form by stimulating the otherwise low intrinsic hydrolysis activity by many orders of magnitude (Fig 2A). Like heterotrimeric G-proteins, Ras proteins are also uniformly

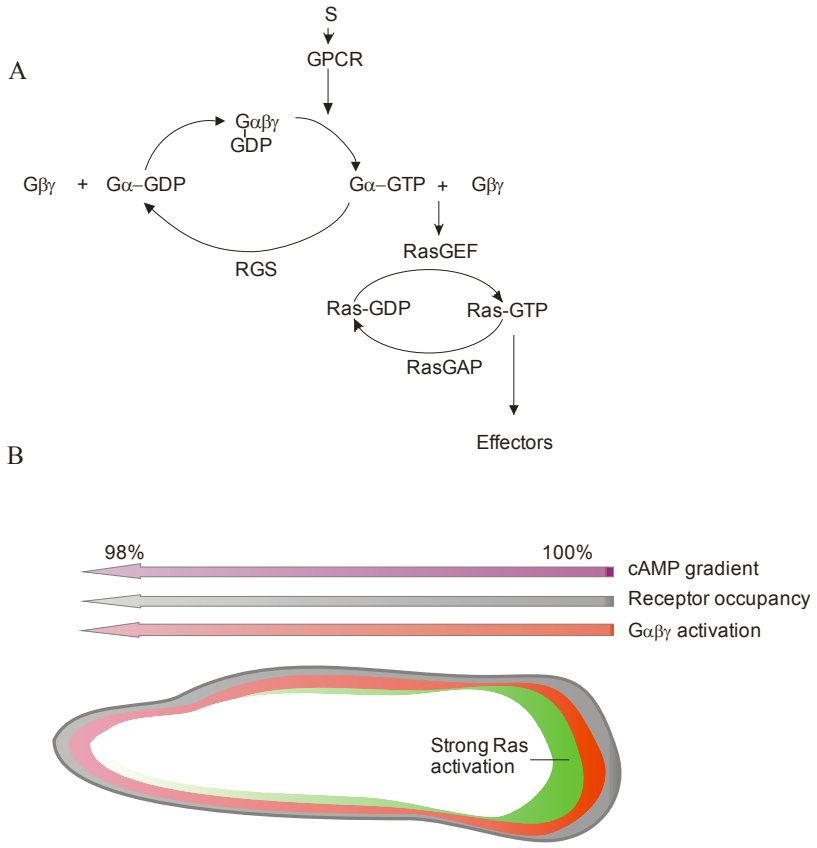

Figure 2 The G-protein cycle (A) Both heterotrimeric and monomeric G-proteins switch between the inactive GDP-bound state and the active GTP-bound state. For heterotrimeric G-proteins, GPCRs (G-protein coupled receptors) act as GEFs (Guanine nucleotide exchange factors) catalyzing the exchange of GDP to GTP. The RGS proteins (Regulators of G-protein cycle) assist the intrinsic GTPase activity of Gα proteins to hydrolyze the bound-GTP and return to the inactive state. RasGEFs and RasGAPs assist the Ras cycle in a similar fashion. The G-proteins interact with the downstream effectors in the active GTP-bound state. (B) Receptor occupancy and the activation of associated heterotrimeric G-proteins are proportional to the steepness of extracellular chemoattractant gradient; which differs 2% across the length of the cell. Due to internal amplification, the activation of Ras is much stronger at the front as compared to the rear of the cell.

Table 1 Gα- proteins in *Dictyostelium*

Gα-proteins in *Dictyostelium*	Expression profile	Function	Ref
Gα1	expressed predominantly in anterior prestalk cells, expression becomes higher during mound stage and continues till culmination stage.	controls prestalk morphogenesis, regulates cGMP pathway, OE cells are large & multinucleate.	[57,58] [59]
Gα2	expressed at low levels during growth & induced to high levels during aggregation.	cAMP chemotaxis & relay, activation of adenyly and guanylyl cyclase.	[46,58] [59]
Gα3	expression induced to high level at 2 h of starvation, then gradually declines	cAMP production, inhibits PKA activity	[44,60] [61]
Gα4	expressed during growth, getting higher at mound stage n continuing till culmination.	folate chemotaxis, late development	[45,47] [62]
Gα5	same as temporal expression of Gα4	inhibit folate chemotaxis, tip morphogenesis	[42,63] [64]
Gα6	expressed during growth and early development		
Gα7	expressed during aggregation and mound stage	Gα7 OE form abnormal fruiting bodies	[65]
Gα8	expressed at low level during growth, reaching maximum at aggregation and mound stage	cell proliferation & differentiation cell-substrate adhesion, proliferation inhibiting activity of AprA.	[65,66] [67,68]
Gα9	expressed throughout growth & development, reach maximum after 5 hours of starvation	delays the expression of cAMP regulated genes & inhibits the formation of cAMP initiation centres during early development.	[39]

distributed along the plasma membrane and are exclusively localized at the leading edge in a chemotaxing cell [56,69]. Contrary to activation of Gαβγ which is proportional to the steepness of gradient, activation of Ras is much stronger as a result of internal amplification (Fig 2B). For such a simple organism, *Dictyostelium* contains an unusually high amount of Ras subfamily proteins; it has 11 Ras proteins, 3 Rap proteins (RapA, B, and C) and one Rheb protein [69,70]. Ras subfamily proteins are involved in many important and diverse processes like growth, chemotaxis, development, cytokinesis and regulation of cytoskeleton (Table 2).

Table 2 Ras proteins in *Dictyostelium*

Protein	Expression profile	Function	Ref
RasB	expressed predominantly during growth and first 8 hours of development	regulation of cell cycle	[71,72]
RasC	expressed throughout life cycle	cAMP relay, development, activation of ACA and PKB	[73,74] [75,76]
RasD	expressed during growth, reappear in tight aggregate stage	phototaxis, thermotaxis cAMP production	[77,78]
RasG	expressed during growth and early development	growth, chemotaxis & development	[76,79] [80,81]
RasS	expressed during starvation and aggregation	regulates the balance between eating and moving	[82,83] [84]
RapA	expressed at all stages but maximally during aggregation and culmination	growth, chemotaxis and cytoskeleton rearrangement	[85,86] [87,88]

RasC and RasG are the best characterized small G-proteins and together important for chemotaxis and development [55,76,81]. RasC is important for proper adenylyl cyclase and PKB activation, which is essential for cAMP relay and development [73,76]. *rasC*-null cells have reduced chemotaxis and abolished aggregation. Cells lacking *rasG* have growth defect, strongly reduced chemotaxis, delayed aggregation and the aggregate size is smaller than wild type cells. cAMP induced accumulation of cGMP and phosphorylation of PKB is also reduced in *rasG*-null cells [80],[81]. Disruption of both *rasC* and *rasG* leads to a complete loss of cAMP relay and

chemotaxis, suggesting that RasC and RasG are together responsible for activating all major downstream pathways [81].

Regulation of the Ras cycle

Recent studies have given important insight in the network regulating Ras activity; however the exact pathways remain largely unknown. In the *Dictyostelium* genome, there are 25 genes encoding RasGEFs and 17 genes for putative RasGAPs [89]. Disruption of the GAP, *nfaA*, results in severe chemotaxis defects, delayed aggregation, increased levels of Ras activation and F-actin. NfaA regulates both RasB and RasG [90]. RasGEFA is essential for the activation of RasC [55,76]. *gefA*$^{-}$ cells are completely lacking RasC activation, and consistently have normal growth and chemotaxis, whereas activation of ACA and development are severely impaired [74,81,91]. RasGEFR specifically activates RasG *in vitro* [55]. Although, *gefR*$^{-}$ cells have reduced cAMP mediated RasG activation, there is still a significant level of active RasG, suggesting there have to be additional RasG specific GEFs.

Since Ras signaling is essential for chemotaxis, cAMP relay and development, it will be essential to further unravel the complex network of GEFs and GAPs that regulate the spatial and temporal activation of Ras.

Developmental pathways

Upon starvation, the aggregation of *Dictyostelium* cells is driven by the synthesis and secretion of cAMP in an oscillatory manner and the ability of cells to sense and respond to it. This is followed by signal relay which involves the activation of Adenylyl Cyclase A (ACA) and subsequent secretion of cAMP [92] which is mainly regulated by RasC via activation of TORC2 pathway [93,94]. Activated TORC2 phosphorylates PKBR1 and PKBA which together play a critical role in signal relay by stimulating ACA [93] (Fig 3). TORC2 is a multi-subunit complex comprising of TOR kinase, PIA, RIP3 and Lst8 [95]. The *rip3*$^{-}$, *pia*$^{-}$and *lst8*$^{-}$ cells fail to aggregate upon starvation and accumulate less ACA [95-97], thereby, strengthening the role of TORC2 complex in development [95].

Dictyostelium genome encodes only two extracellular-signal related kinases sharing 37% homology: ERK1 and ERK2, which are expressed during growth and throughout development [98-100]. ERKs are involved in many vital cellular processes including cell proliferation, gene

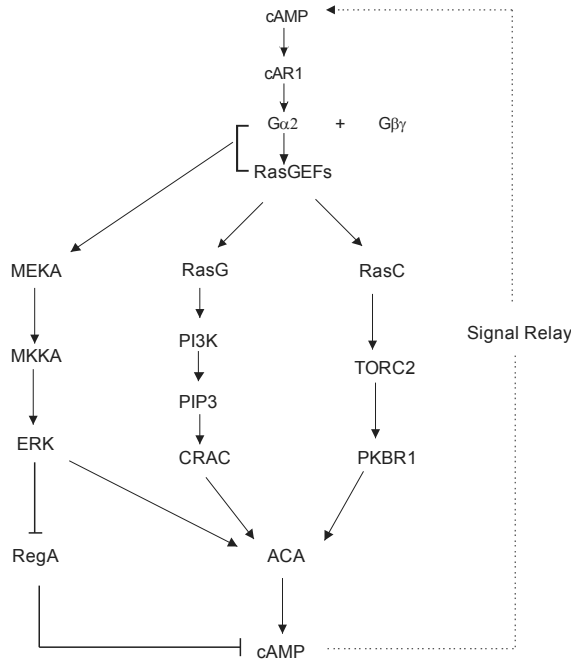

Figure 3 Signaling pathways regulating development The activation of heterotrimeric G-proteins by binding of cAMP to cAR1 leads to activation of Ras proteins and MAP kinase pathways. The activation of RasG, RasC and MEKA leads to recruitment of PI3K, TorC2 and ERK respectively, which is important for activation of adenylyl cyclase A (ACA) and hence cAMP relay. ERK2 inhibits the cAMP specific phosphodiesterases RegA leading to increased levels of cAMP important for cell aggregation.

expression, growth, differentiation and apoptosis [101]. In other organisms, signal transduction to ERKs is considered to be regulated by Raf kinases which are activated by Ras proteins via a kinase cascade consisting of MAPK/MEK/ERK in mammalian system [101-103]. However recent studies revealed a Ras-independent mechanism for ERK activation [104,105]. *Dictyostelium* ERKs are a part of kinase cascade that includes MEKA and MKKA. MEKA is required for normal chemotaxis to cAMP, accumulation of guanylyl cyclase in response to cAMP and for aggregation [106]. MKKA regulates morphogenesis, developmental timing and cell-specific patterning during multicellular development [107]. *erk1*-null cells form small aggregates which may or may not form tips and the ones which succeed to form tips and

complete the development cycle have smaller fruiting bodies. ERK2 play a minor role in chemotaxis to cAMP and folate and is also involved in cell differentiation and development of prespore cells [108,109]. Moreover, ERK2 is also known to regulate cAMP levels through RegA, a cAMP-specific phosphodiesterases [110] (Fig 3). The involvement of ERK signalling in growth and development necessitates the study to find other components in order to gain more insight into this pathway.

Chemotaxis pathways

Ras regulates chemotaxis by activating various downstream effector proteins (Fig 4). One of the most studied downstream effectors of Ras is PI3K. Ras activates the basal PI3K present at the membrane; through a positive feedback loop via F-actin polymerization, cytosolic PI3K is also recruited at the membrane [56]. *Dictyostelium* has five class I PI3K proteins [111], which all consist of a characteristic Ras-binding domain that specifically interact with GTP-bound Ras proteins [112]. In a chemoattractant gradient PI3K is localized at the leading edge where it catalyzes the production of PIP3 from PI(4,5)P2 while PTEN is localized at the lateral and posterior sides of the cell catalyzing the converse reaction of dephosphorylating PIP3 to PIP2. Due to this reciprocal localization and activity of these two proteins, a PIP3 gradient is formed promoting pseudopod extension at the leading edge whereas PIP2 suppresses lateral pseudopods. *pten*-null cells can sense the direction of gradient and become polarized but they exhibit inefficient chemotaxis with decreased chemotaxis index, speed and directional persistence [113]. Although these initial studies suggested that the PIP3 pathway is essential and acts as a molecular compass for chemotaxis [114,115], recent data suggest that PI3K only plays an important role in directional sensing of shallow gradients [116,117]. Cells with disruptions in all five *pi3k* genes were still capable of performing normal chemotaxis [111,118] in steep gradients, albeit with a reduced speed, indicating the presence of other pathways that mediate directional sensing. A pathway mediating chemotaxis in the absence of PI3K was identified that involved Phospholipase A2 (PLA2) [116,119]. Although not much is known about this pathway apart from the fact that it is regulated by Ca^{2+} uptake and cytosolic Ca^{2+}, however, various studies suggest that PLA2 is redundant for chemotaxis in steep gradients [116,120]. The cells lacking *pi3k* and *pla2* when starved for 7 hours were still able to exhibit chemotaxis suggesting the existence of still some other pathway. Mutational analysis revealed the importance of sGC

pathway in regulating chemotaxis [121]. sGC is localized at the leading edge and is predominantly enriched in the extended pseudopod where it interacts with actin filaments serving as the memory of an existing pseudopod. It also catalyzes the formation of cGMP by hydrolyzing GTP [122]. cGMP induces the formation of myosin filaments thereby also inhibiting the formation of pseudopods at the rear and at lateral sides of the cell [123,124]. RasG regulates the production of cGMP upon cAMP stimulation further signifying the importance of Ras in chemotaxis [81]. Also RasC activates TORC2 which then phosphorylates PKBR1 and PKBA at the leading edge of cell thereby regulating chemotactic responses [93].

Role of Ras in Cytoskeleton Rearrangement

Symmetry breaking in the above described signalling pathways leads to major changes in the cytoskeleton; actin polymerization occurs at the leading edge of the cell, while acto-myosin filaments are formed at the rear and back of the cell. Since a properly organized cytoskeleton is essential for chemotaxis and development, regulation of cytoskeleton is of utmost importance. Actin polymerization at the leading edge is mediated by the Arp2/3 complex which produces a complex network of branched filaments that leads to pseudopod extension. The regulation of this process involves several groups of proteins, including the Rho family of small G-proteins, WASP (Wiskott-Aldrich syndrome protein), and WAVE/SCAR [125-127].

At the lateral and posterior sides of a chemotaxing cell, pseudopod extension is suppressed by assembly of myosin II filaments [128-130] in a cGMP dependent manner [85]. Myosin II is responsible for contraction of rear end as well as involved in spatio-temporal regulation of traction forces [131,132]. Furthermore, at the leading edge activated RapA stimulates Phg2 (a serine/threonine kinase) which phosphorylates and thus leads to disassembly of myosin II [133-135]. Disruption of myosin II leads to inefficient chemotaxis and formation of lateral pseudopodia [136,137].

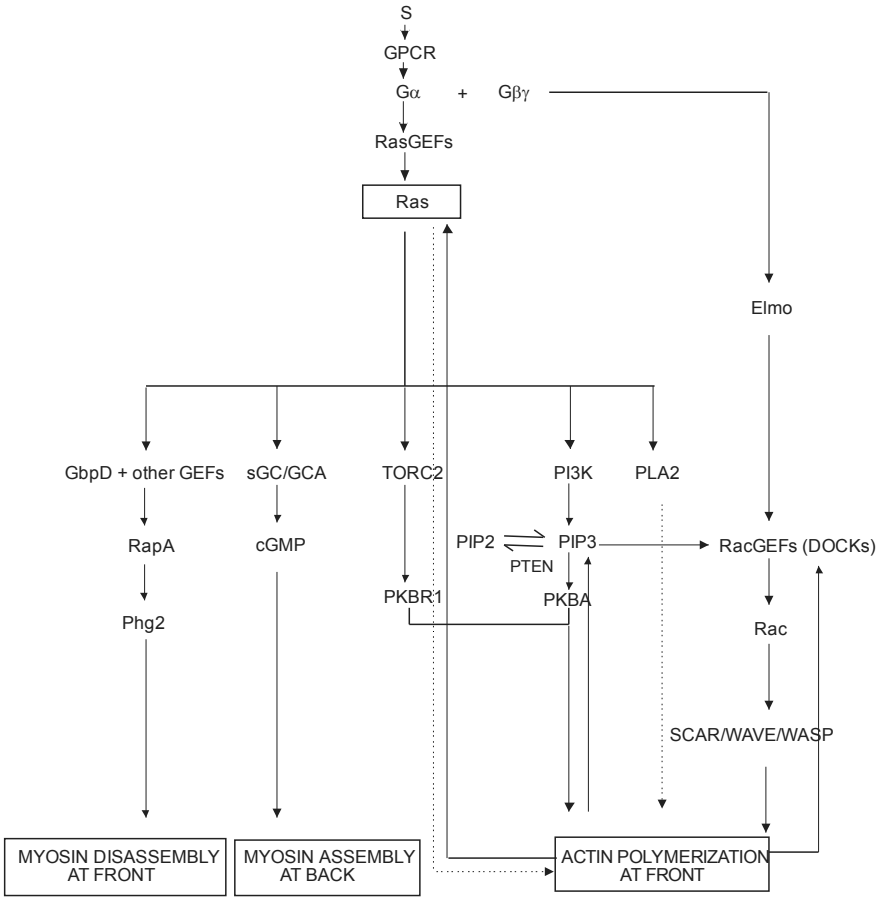

Figure 4 Signaling pathways regulating chemotaxis Binding of chemoattractant to the receptor leads to the activation of associated heterotrimeric G-protein which further results in rapid activation of Ras at the leading edge. The activation of Ras results in the activation of four signaling pathways along with Rap specific GEFs. The activated PI3K and TorC2 are important for actin polymerization at the leading edge. Rac proteins also play an important role in actin polymerization at the front of the cell. cGMP produced by sGC and GCA is a key regulator of myosin filament formation at the back of the cell. Upon activation, Rap binds to Phg2 and inhibits formation of myosin filaments at the front of the cell. The presence of several positive feedback loops results in stronger Ras activation and F-actin polymerization at the front leading to efficient chemotaxis. The dotted lines indicate pathways that are not completely characterized yet.

Aim of the thesis

Chemotaxis is a complex cellular process involving several interconnected signaling pathways, positive and negative feedback loops, multiple regulators and effector proteins. The information presented in this chapter shows that processes involved in chemotaxis are very complicated. In Chapter 2 of the thesis we show that cells lacking all four known chemotaxis pathways downstream of Ras: sGC, PLA2, PI3K and TORC2, are still able to move. Thus we deduced the basal module for chemotaxis detailing the minimal requirements. Subsequently we characterize Ras activation, symmetry breaking and confinement during *Dictyostelium* chemotaxis using various mutant cell lines in Chapter 3. Nevertheless, in addition to these important components, it is likely that there are many additional proteins, functioning as scaffold, activators, inhibitors and/or effectors in the chemotaxis pathway that still need to be identified. To discover these additional components of the presented basal pathway is a challenging task. Chapter 4 details a new proteomic approach undertaken to find the novel players in chemotactic signal transduction. Ric8, which we found in the screens of several Gα proteins, was selected for further studies and shown to be acting as a non-receptor GEF for *Dictyostelium* Gα proteins discussed in detail in Chapter 5. Similarly, Chapter 6 deals with characterizing a leucine rich repeat protein LrrA which could be a putative link between Gα and Ras signaling.

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